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# Characterization of Natural Epstein-Barr Virus Infection and Replication in Smooth Muscle Cells From a Leiomyosarcoma

**Hal B. Jenson,[1–3]* Eduardo A. Montalvo,[1,4] Kenneth L. McClain,[5] Yasmin Ench,[1] Patty Heard,[1] Barbara A. Christy,[4] Pamela J. Dewalt-Hagan,[6] and Mary Pat Moyer[2,3,6]**

[1]*Department of Pediatrics, The University of Texas Health Science Center at San Antonio, San Antonio, Texas*
[2]*Department of Microbiology, The University of Texas Health Science Center at San Antonio, San Antonio, Texas*
[3]*The San Antonio Cancer Institute, The University of Texas Health Science Center at San Antonio, San Antonio, Texas*
[4]*Department of Molecular Medicine at the Institute of Biotechnology / Center for Molecular Medicine, The University of Texas Health Science Center at San Antonio, San Antonio, Texas*
[5]*Department of Pediatrics, Baylor University School of Medicine, Houston, Texas*
[6]*Department of Surgery, The University of Texas Health Science Center at San Antonio, San Antonio, Texas*

Cells from a leiomyosarcoma tumor (LMS-1) from a patient with the acquired immunodeficiency syndrome (AIDS) were explanted, cultured in vitro, and studied by phase-contrast microscopy for morphologic and growth characteristics, immunostaining for cell markers, EBER in situ hybridization and polymerase chain reaction for detection of Epstein-Barr virus (EBV), and immunostaining for expression of EBV antigens. The cells exhibited very slow growth in vitro, with unusual elliptical and spindle-shaped morphology and fragmentation of the cytoplasm into long, tapering, cytoplasmic processes. Greater than 90% of cells expressed diffuse distribution of the smooth muscle isoform of actin by immunoperoxidase staining. Approximately 25% of cells expressed very bright fluorescence by immunostaining of the smooth muscle isoforms of calponin and actin. The majority of cells demonstrated a weak signal for CD21; approximately 5–10% of cells showed a strong signal that was confined to cell surfaces. The cultured cells harbored EBV, and infectious EBV continued to be detected by polymerase chain reaction and virus culture through several passages in vitro. Several EBV antigens were expressed, including latent antigen EBNA-1, immediate-early antigen BZLF1, early antigen EA-D, and late antigens, including viral capsid antigen p160, gp125, and membrane antigen gp350. Human umbilical cord lymphocytes that were transformed with virus isolated from cultured cells yielded immortalized cell lines that expressed EBV antigens similar to other EBV-transformed lymphocyte cell lines. These results confirm that EBV is capable of lytic infection of smooth muscle cells with expression of a repertoire of latent and replicative viral products and production of infectious virus. EBV infection of smooth muscle cells may contribute to the oncogenesis of leiomyosarcomas. ***J. Med. Virol. 57:36–46, 1999*** © 1999 Wiley-Liss, Inc.

**KEY WORDS:** human immunodeficiency virus; acquired immunodeficiency syndrome-associated malignancy; leiomyosarcoma

## INTRODUCTION

Leiomyosarcomas, malignant tumors of smooth muscle origin that also are known as spindle cell tumors, are very rare tumors in immunocompetent individuals. These uncommon tumors in adults usually develop in the uterus and develop in children as visceral leiomyosarcomas at a rate of approximately one case per 10 million children [Botting et al., 1965; Lack, 1986; Yannopoulos and Stout, 1962]. Immunocompromised individuals have a higher overall incidence of malignancies than immunocompetent individuals. The majority of neoplasms in immunocompromised individuals are skin cancers and lymphomas, with the frequent occurrence of unusual tumor types [Penn, 1995]. Leiomyosarcomas have been reported with increased

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*Correspondence to: Hal B. Jenson, M.D., Department of Pediatrics, University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, TX 78284-7811. E-mail: jenson@uthscsa.edu

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frequency in patients receiving anticancer or antirejection medication for organ transplantation [Ha et al., 1993; Shen and Yunis, 1976; Swanson et al., 1991]. An unusually high incidence, approximately one case per 5,000 children, of smooth muscle tumors (leiomyomas and leiomyosarcomas) has been found in children with the acquired immunodeficiency syndrome (AIDS) caused by human immunodeficiency virus (HIV) infection [Chadwick et al., 1990; Levin et al., 1994; McLoughlin et al., 1991; Orlow et al., 1992; Ross et al., 1992; van Hoeven et al., 1993]. Leiomyosarcomas are the second most frequent tumor among children with AIDS. In contrast to leiomyosarcomas from HIV-uninfected persons, all cells of leiomyosarcomas from HIV-infected persons harbor Epstein-Barr virus (EBV), with an average of 4.5 EBV genomes per cell, and also express CD21 (CR2), the cellular receptor for EBV [Jenson et al., 1997b; McClain et al., 1995]. The EBV infection is clonal, indicating that EBV infection precedes malignant transformation [McClain et al., 1995].

Cells from a leiomyosarcoma of a young person with AIDS were explanted and cultured in vitro. These cells were studied under phase-contrast microscopy and were examined for morphologic growth characteristics, phenotypic cell markers, the presence and replication of EBV, cellular expression of EBV products, and the production of infectious EBV.

## MATERIALS AND METHODS

### Cells

Cells were explanted from a leiomyosarcoma, which was designated LMS-1, of the thoracic spine from a 29-year-old Native-American female who was diagnosed with HIV-1 infection at the age of 27 years after heterosexual HIV-1 transmission. A 0.3 $mm^3$ specimen of fresh tumor was minced and washed five times in virus transport medium composed of Minimal essential medium with Earle's salts (Gibco BRL Life Technologies, Grand Island, NY), 1% Glutamax-1 (Gibco BRL Life Technologies], 2% penicillin/streptomycin/Fungizone (JRH Biosciences, Lenexa, KS), and 100 μl/ml gentamicin (Sigma, St. Louis, MO). Explants were plated into flasks by using M3:10 growth medium (In-Cell, San Antonio, TX) supplemented with 10% fetal bovine serum (HyClone, Logan, UT). Cells were passaged after washing twice with calcium- and magnesium-free phosphate-buffered saline (PBS) followed by 0.25% trypsin (Mediatech, Herndon, VA). Cells were examined under phase-contrast microscopy by using an Olympus CK2 phase-contrast inverted microscope (Tokyo, Japan).

The EL-1 cell line was established by immortalization of human umbilical cord lymphocytes with virus from the cultured LMS-1 cells. A 1-ml aliquot of filtered (0.22 μm) supernatant from LMS-1 cells was absorbed overnight with $2 \times 10^6$ human umbilical cord lymphocytes that were isolated by layering heparinized blood on Histo-Paque 1077 (Sigma) [Jenson et al., 1997a].

Smooth muscle cell lines that were studied for comparison with LMS-1 cells included the HA-VSMC cell line, a continuous smooth muscle cell line derived from the aorta of an 11-month-old Caucasian child (CRL-1999; American Tissue Culture Collection; Rockville, MD), and the A7r5 cell line, a continuous clonal cell line derived from smooth muscle of embryonic thoracic aorta of DB1X rat (CRL-1444; American Tissue Culture Collection) [Kimes and Brandt, 1976].

### Immunohistochemistry for Phenotypic Cell Markers

Cultured LMS-1 cells were grown on LabTek slides (Nunc, Naperville, IL), fixed with cold acetone (4°C), and assayed for cell-type and differentiation markers by using standard immunohistochemistry methods. Cell markers were tested by using monoclonal antibodies against galactocerebroside (1-351-621; Boehringer Mannheim, Indianapolis, IN); CD4 and CD23 (Oncogene Science, Manhasset, NY); vimentin (M0725; DAKO, Carpenteria, CA), smooth muscle actin (M0851; DAKO), smooth muscle myosin (M7786; Sigma), skeletal muscle myosin (M7523; Sigma), factor VIII (M0616; DAKO), glial fibrillary acid protein (M0761; DAKO), and neurofilament (M0762; DAKO) (Table I). Cells were incubated with primary monoclonal antibodies of the desired specificity at the dilution recommended by the manufacturer and were stained with secondary biotinylated immunoperoxidase antibodies (DAKO). Percentages of expression for each marker were calculated as the mean of observations of at least ten low-power microscopic fields.

Cultured LMS-1 cells were assayed for expression of the smooth muscle markers calponin and smooth muscle actin by immunofluorescence staining of cells grown on sterile glass coverslips, rinsed in PBS, fixed in 4% paraformaldehyde for 8 minutes at room temperature, rinsed twice in PBS, incubated in 0.1% NP40 in PBS for 15 minutes at room temperature, rinsed extensively in PBS, incubated in 10% normal goat serum in PBS for several hours to overnight at 4°C, rinsed in 1% bovine serum albumin (BSA) in PBS, and incubated with primary antibody (1:500 for anti-calponin, 1:400-1:1,000 for anti-α smooth muscle actin) several hours to overnight at 4°C. Cells were rinsed thoroughly in 1% BSA in PBS and incubated with 1:500 fluorescein-conjugated goat antimouse immunoglobulin (Ig) G (Southern Biotechnology Associates, Birmingham, AL) for 45 minutes at room temperature. Cells were rinsed thoroughly in 1% BSA in PBS, stained with 4,6-diamidino-2-phenylindole (DAPI) to visualize nuclei, rinsed in water, and mounted onto glass slides with aqueous mounting medium (PermaFlour; Immunon, Pittsburgh, PA). The primary antibody for calponin was a mouse IgG1 monoclonal antibody to human calponin (C-2687; Sigma) and, for smooth muscle actin, it was a mouse IgG2a monoclonal antibody to the N-terminal region of α smooth muscle actin (A-2547; Sigma). The CV-1 cell line, an African green monkey fibroblastic cell line, was used as a negative control.

TABLE I. Cell Surface Markers of the LMS-1 Leiomyosarcoma Cells Cultured in Vitro

| Cell surface marker (method) | Percentage of positive cells |
|---|---|
| Smooth muscle cell markers | |
| Smooth muscle myosin (immunoperoxidase) | >90% |
| Calponin (immunofluorescence) | 25% (very bright) |
| Smooth muscle actin (immunoperoxidase) | >90% |
| Smooth muscle actin (immunofluorescence) | 25% (very bright) |
| Skeletal muscle cell marker | |
| Myosin heavy chain (immunoperoxidase) | Negative |
| Mesenchymal cell marker | |
| Vimentin (immunoperoxidase) | >90% |
| Endothelial cell marker | |
| Factor VIII (immunoperoxidase) | ⩽1% |
| Neural cell markers (immunoperoxidase) | |
| Galactocerebroside (GalC) | ⩽1% |
| Glial fibrillary acid protein | Negative |
| Neurofilament | Negative |
| Lymphoid cell (immunoperoxidase) | |
| CD21 (CR2; EBV receptor)[a] | |
| OKB7 | Most weakly positive; ~5–10% strongly positive |
| HB5 | Negative |
| B2 | Negative |
| CD4 (T4 lymphocyte) | Negative |
| CD23 | Negative |

[a]EBV, Epstein-Barr virus.

### Immunofluorescence for EBV CD21 Cell Receptor

Three different monoclonal antibodies were used to assay LMS-1 cells for expression of the CD21 receptor: OKB7 (Ortho Diagnostic Systems, Raritan, NJ) [Nemerow et al., 1985], HB5 (Becton-Dickinson Immunocytometry Systems, San Jose, CA) [Favrot et al., 1986], and B2 (Coulter Diagnostics, Hialeah, FL). Mouse isotype monoclonal antibodies were used as negative control antibodies.

LMS-1 cells were grown on sterile, eight-chambered glass slides (SuperCell; Fisher Scientific, Pittsburgh, PA), fixed in cold acetone for 5 minutes, and stored at −20°C until they were assayed. Two indirect immunofluorescent assay methods were used. In the first method, slides were incubated with diluted monoclonal antibody (OKB7; 1:10 dilution in Dulbecco's PBS; HB5 diluted 1:5 or B2 diluted 1:10) for 90 minutes followed by diluted (1:50) goat $F(ab')_2$ antimouse Ig (gamma- and light-chain specific) fluorescein isothiocyanate (FITC)-conjugated antibody (Biosource International, Camarillo, CA) for 30 minutes. In the second method, slides were incubated with diluted OKB7 or HB5 for 1 hour followed by incubation with biotinylated horse antimouse IgG (gamma chain specific) affinity-purified antibody (Vector Laboratories, Burlingame, CA) at a 1:20 dilution in PBS followed by fluorescein-streptavidin (Vector Laboratories) at a 1:100 dilution in PBS. Evans blue counterstain was used in both methods. Stained slides were covered with a coverglass and glycerol mounting medium (glycerol:PBS, 9:1) containing 0.025% DABCO (Sigma) and viewed with a Zeiss epifluorescent microscope (Thornwood, NY) with filters for fluorescein. Positive control cells included SVK-CR2 [Li et al., 1992] cells as strongly positive, Raji cells [Pulvertaft, 1965] as moderately positive, and Molt-4 [Minowada et al., 1972] cells as weakly positive cell lines. Negative control cells were SVK-Neo and HSB-2 cells [Li et al., 1992; Royston et al., 1974].

### EBER In Situ Hybridization

Cytospins of cultured LMS-1 cells were fixed on glass slides with methanol for 15 minutes and were hybridized with biotinylated oligonucleotides complementary to three regions of the EBV EBER 1 gene [Chang et al., 1993]. Detection was performed with the DETEK HRP Signal Generating System (Enzo Diagnostics, Farmingdale, NY).

### Polymerase Chain Reaction Amplification

Detection of EBV DNA in cultured cells was performed by polymerase chain reaction (PCR) amplification using primers (5′-GGTCCATCATCTTCAGCAAAG-3′ and 5′-CATGCATATTTCAACTGGGC-3′) that amplify a 269-bp fragment corresponding to −221 to +45 bp of the EBV BZLF1 promoter. Total intracellular DNA for PCR was prepared from $10^6$ cells by centrifugation, washed once with PBS, and suspended in a lysing solution of 0.5 M NaCl; 0.05 M EDTA; 0.1 M Tris, pH 8.0; 10% sodium dodecylsulfate; and 20 mg/ml proteinase K. The DNA samples were extracted with phenol/chloroform, precipitated in ethanol, and resuspended in 0.1 M Tris, pH 8.0, 0.001 M EDTA. The DNA was amplified in a 20 μl volume with 10 ng genomic DNA; 0.5 mM of each dNTP; 400 nmole of each primer; 50 mM KCl; 10 mM Tris, pH 8.3; 1.5 mM $MgCl_2$; and 2.5 U *Taq* polymerase. The conditions for PCR were 35 cycles at 94°C for 1 minute and at 72°C for 1 minute, with a final incubation at 72°C for 10 minutes. Ampli-

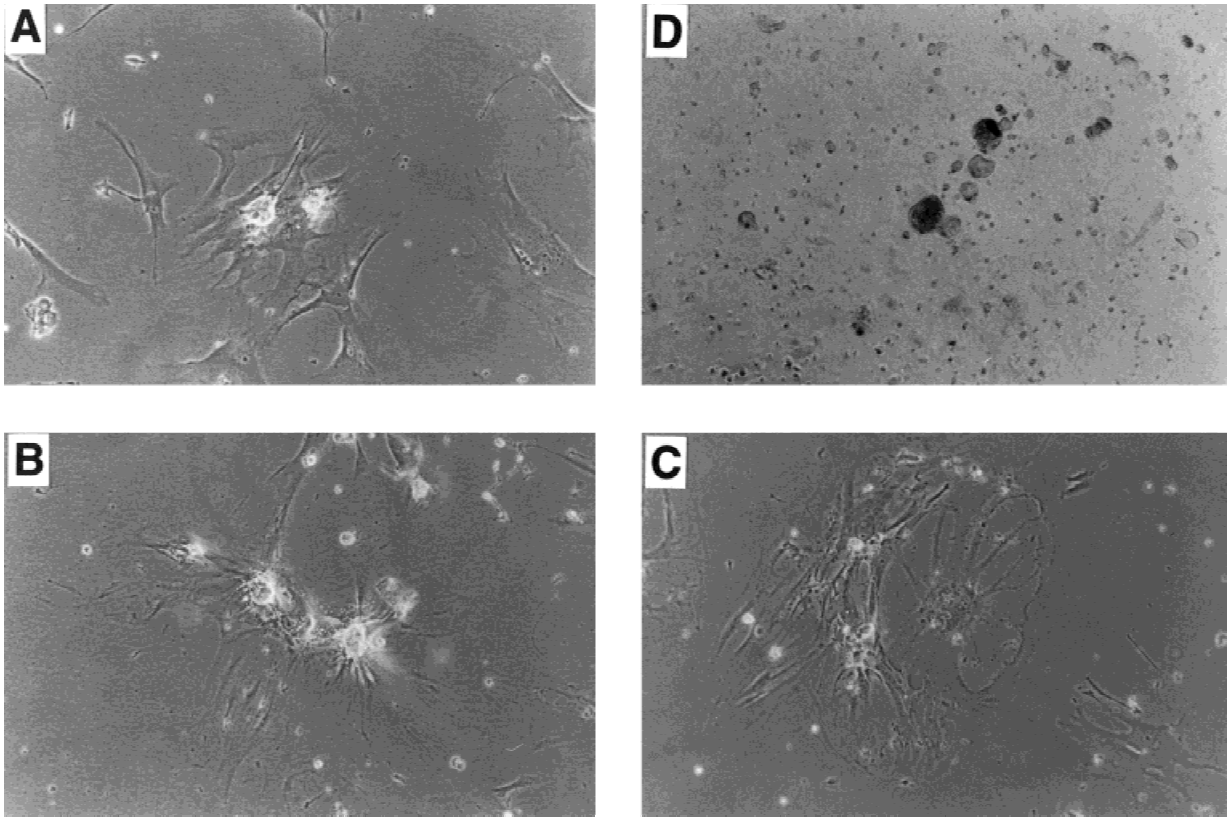


Fig. 1. Phase-contrast microscopy of the LMS-1 leiomyosarcoma cells cultured in vitro. The cells grew slowly as isolated individual cells with unusual morphology. **A:** appearance of monolayer cells at day 9 with large, refractive, cytoplasmic inclusions. **B; C:** Appearance of monolayer cells at 25 days with starburst (B) and wheel-shaped (C) cells. **D:** appearance of a subpopulation of detached cells in suspension at 31 days. Magnification ×120.

fication products were separated by electrophoresis through a 2% agarose gel.

### Immunofluorescence for EBV Antigens

Cultured LMS-1 cells and cell smears of the EL-1 lymphocyte cell line were assayed by immunofluorescence for expression of the EBV antigens EBNA-1, EBNA-2, LMP-1, BZLF1, EA-D, EA-R, viral capsid antigens (VCA), and membrane antigen (MA) as described previously [Jenson et al., 1997a].

## RESULTS

### Phase-Contrast Microscopy

The explanted and cultured cells from the LMS-1 leiomyosarcoma demonstrated unusual individual cell shapes (Fig. 1). By phase-contrast microscopy, the typical cell was elliptical or spindle-shaped, with long, tapering cytoplasmic processes that remained attached to the flask. Many cells had a flat, cylindrical shape with fragmentation of the cytoplasm into long, narrow cytoplasmic processes. In some cells, the ends of these processes appeared to coalesce. The cells initially doubled in approximately 3–5 days for the first passages and were maintained by splitting 1:2 with fresh growth medium. The cells began to grow very slowly though with the doubling time gradually increasing, and approaching 1 month by passage 10. After that, the cells remained live but quiescent. With continued culture, networks of cells appeared to connect by cytoplasmic processes. The central portion of the cells typically expanded with development of vacuoles and refractile rounded inclusions (Fig. 1A), creating star-burst (Fig. 1B) or wheel-shaped cells (Fig. 1C). Continued culture resulted in eventual detachment of the cells (Fig. 1D).

### Phenotypic Cell Markers

Smooth muscle cell calponin (also called h1 or basic calponin) is a thin filament-associated protein that is apparently involved in regulation of smooth muscle contraction. The smooth muscle cell form of calponin is restricted to smooth muscle cells in the adult but is expressed in embryos in the early cardiac tube [Miano and Olson, 1996]. Smooth muscle α-actin also is widely used as a smooth muscle cell marker, although its expression also is detected in skeletal and cardiac muscles to a lesser degree [Ruzicka and Schwartz, 1988]. Approximately 25% of LMS-1 cells expressed very bright fluorescence to smooth muscle calponin as well as smooth muscle α-actin (Fig. 2) at levels comparable to the fluorescence observed in HA-VSMC cells and A7r5 cells (Table I). CV-1 cells were used as a

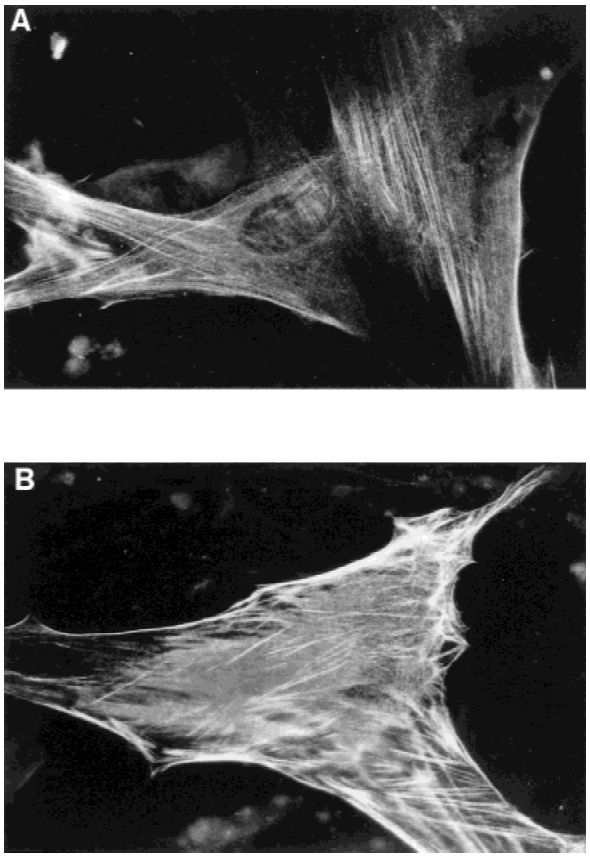


Fig. 2. Detection of calponin **(A)** and smooth muscle actin **(B)** by immunostaining in the LMS-1 leiomyosarcoma cells cultured in vitro. The individual cells demonstrated diffuse distribution of both proteins.

negative control and showed no immunofluorescence for either protein (data not shown). The LMS-1 cells did not express skeletal muscle myosin heavy chain, a skeletal muscle marker (data not shown).

Cell type-specific markers are necessary for validation of cell type and differentiation lineage. Greater than 90% of LMS-1 cells expressed vimentin, a general marker of mesenchymal cells (Table I). The expression of endothelial, neural, and lymphoid cell markers was also analyzed. Factor VIII, an endothelial cell marker that would be expected in endothelial cells or in a mixed or multilineage mesenchymal population with endothelial differentiation potential, was undetectable in cultured LMS-1 cells. Similarly, less than 1% of the cells expressed the neural cell markers galactocerebroside (GalC) glycolipid, glial fibrillary acid protein, and neurofilament protein. Two lymphoid cell markers, CD4 and CD23, were also undetectable in LMS-1 cells.

Another lymphoid cell marker, CD21 (also known as CR2), serves as the receptor for EBV on B lymphocytes and is also the receptor for the C3d component of complement [Hutt-Fletcher et al., 1983; Jondal et al., 1976]. Immunofluorescence of cultured LMS-1 cells for CD21 using the OKB7 monoclonal antibody [Nemerow et al., 1985] to CD21 detected generally weak fluorescence that was confined to the surface of the cells and around vacuoles (Fig. 3A). Using a biotin-streptavidin procedure, the majority of LMS-1 cells showed weak immunofluorescence, with a strong signal in approximately 5–10% of cells primarily on cell surfaces and in the strands connecting cells (Fig. 3B). Immunofluorescence for CD21 using the HB5 monoclonal antibody [Fingeroth et al., 1984] was undetectable by both methods (data not shown). The LMS-1 cells were negative for immunofluorescence using B2 antibody (IgM monoclonal antibody specific for a B lymphocyte antigen), and mouse IgG and IgM isotype antibodies.

### Detection of EBV EBER and DNA in Cultured LMS-1 Cells

We have previously shown by in situ hybridization of tumor sections that muscle cells of both leiomyosarcomas and leiomyomas from HIV-infected patients harbor EBV [Jenson et al., 1997b; McClain et al., 1995] in contrast to these types of tumors from HIV-uninfected patients. Studies were performed using EBER in situ hybridization and PCR primers specific to the EBV BZLF1 promoter to determine whether EBV was present in explanted and cultured LMS-1 cells. EBER is present in all forms of EBV latency [Tierney et al., 1994]. EBER was detected in all LMS-1 cells at passage 3, with a strong signal in at least 50% of cells and weak detection in the remaining cells, similar to EBER results of the original leiomyosarcoma tumors [McClain et al., 1995]. By passage 5, the EBER signal was negative (data not shown).

The BZLF1 region for PCR was selected because the specificity of these primers for EBV has been confirmed by DNA sequencing of the PCR products in studies of the BZLF1 promoter (Figure 4 and unpublished data). The expected 269-bp, EBV-specific fragment was detected easily in the cultured LMS-1 cells (Fig. 4). These results are consistent with previous findings of EBV infection of these tumor cells using PCR primers for the *Bam*HI W (internal repeat 1) region of EBV in the original leiomyosarcoma tumor [McClain et al., 1995].

### EBV Gene Expression in Cultured LMS-1 Cells

Lymphoid cell lines immortalized by EBV express a limited repertoire of virus-encoded gene products during latent virus infection but with disruption of latency express lytic viral proteins. The expression of various EBV gene products was studied in cultured LMS-1 cells (Fig. 5, Table II). Immunofluorescent detection of EBV antigens in cultured LMS-1 cells demonstrated expression of latent antigens, immediate-early antigens, early antigens, and late antigens. Latent EBNA-1 antigen was detected by using two different monoclonal IgG1 antibodies: one supplied as a culture supernatant (Chemicon, Tumecula, CA) and a second that recognizes the 72-kDa (Raji) and the 87-kDa (wild type) EBNA-1 (Advanced Biotechnologies Incorporated, Columbia, MD). The results with both antibodies were similar. Approximately 3% of trypsinized cells (containing populations derived from both adherent and floating cells) stained positive for EBNA-1, but

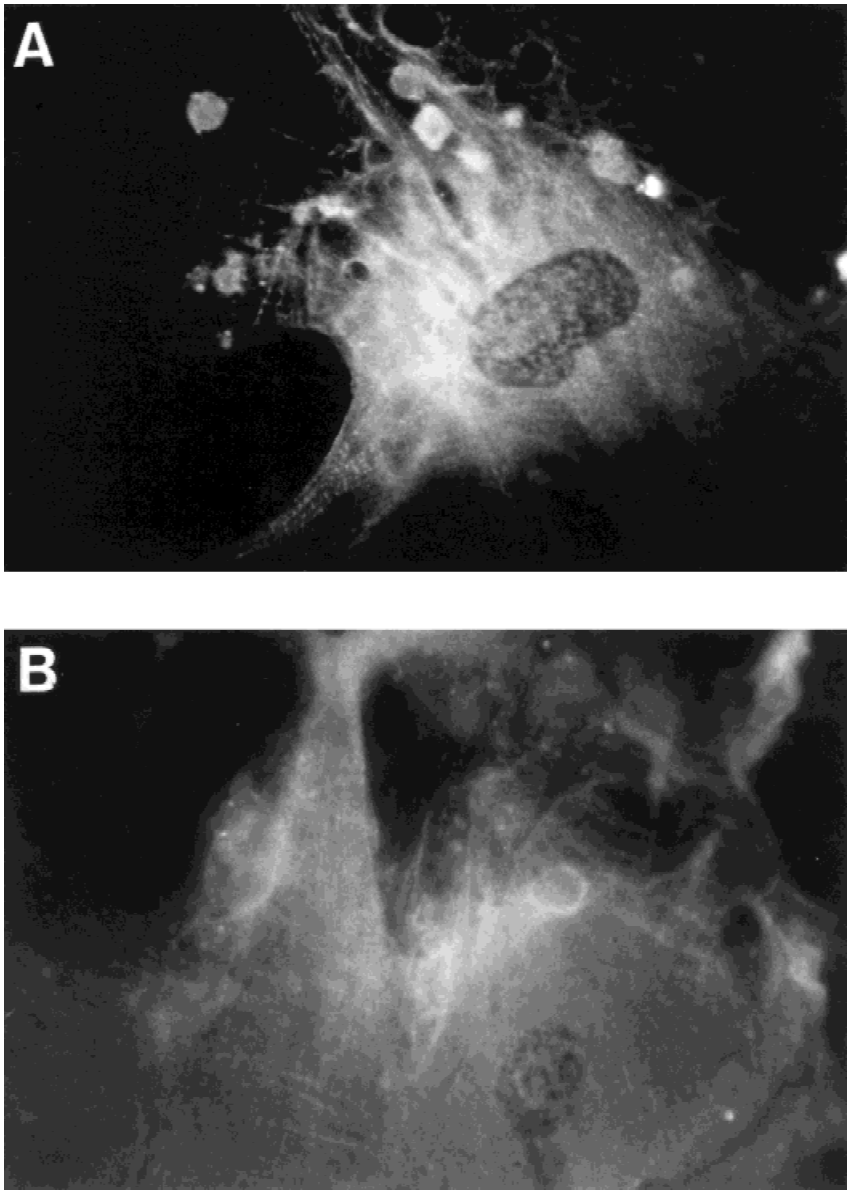


Fig. 3. Expression of the CD21 Epstein-Barr virus (EBV) receptor by immunofluorescence with the OKB7 [Nemerow et al., 1985] monoclonal antibody in the LMS-1 leiomyosarcoma cells cultured in vitro. **A:** A two-step immunofluorescence method detected weak fluorescence that was confined to the cell surfaces, and that is seen as fibers extending between cells and around vacuoles. **B:** A biotin-streptavidin procedure demonstrated weak signal in the majority of cells, with approximately 5–10% of cells showing a strong signal that was confined to cell surfaces, usually in strands. Magnification ×500.

⩽1% of the adherent cells were positive (Fig. 5A). Expression of EBNA-2 and LMP-1 was not detected (data not shown). The immediate-early antigen BZLF1 was detected in ⩽1% of cells (Fig. 5B). Early antigens were detected in ⩽1% of adherent cells, although approximately 20–25% of the older, detached cells were positive for both EA-D (Fig. 5C) and EA-R (data not shown), suggesting that the spread of EBV infection results in loss of adherence of the cells to the inert matrix. The late antigens p160, gp125, and gp350 were also detected in the cultured LMS-1 cells (Fig. 5D–F) indicating complete viral expression and assembly of virions in these cells.

### Transformation of Primary Lymphocytes

Lytic replication of EBV within cultured LMS-1 cells was confirmed by transformation assay of supernatant for infectious EBV. EBV was cultured from the original supernatant from cultured LMS-1 cells and from each passage tested (passages 2 through 6). Human umbilical lymphocytes immortalized by filtered supernatant lymphocytes demonstrated proliferation, aggregation, and growth characteristic of EBV-infected lymphocytes. Immunofluorescence of the resulting immortalized EL-1 lymphocyte cell line demonstrated expression of EBV genes including latent antigens EBNA-1,

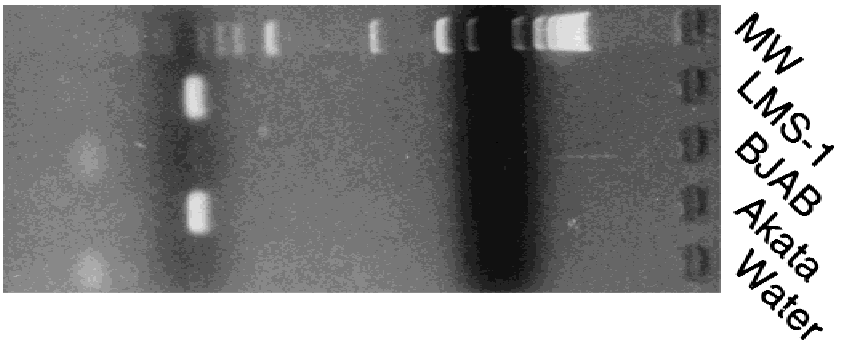


Fig. 4. Detection of EBV genomes by polymerase chain reaction (PCR) amplification using primers for the BZLF1 promoter in the LMS-1 leiomyosarcoma cells cultured in vitro. Cultured LMS-1 cells and the Akata lymphocyte cell line, an EBV genome-positive Burkitt's lymphoma cell line [Takada et al., 1991], demonstrated presence of the 269-base-pair, amplified fragment of EBV DNA. No similar DNA fragment was detected in the BJAB cell line, an EBV genome-negative B cell line that was included as a negative control. A sample without any intracellular DNA (labeled water) was also included as a negative control. MW = molecular weight (markers).

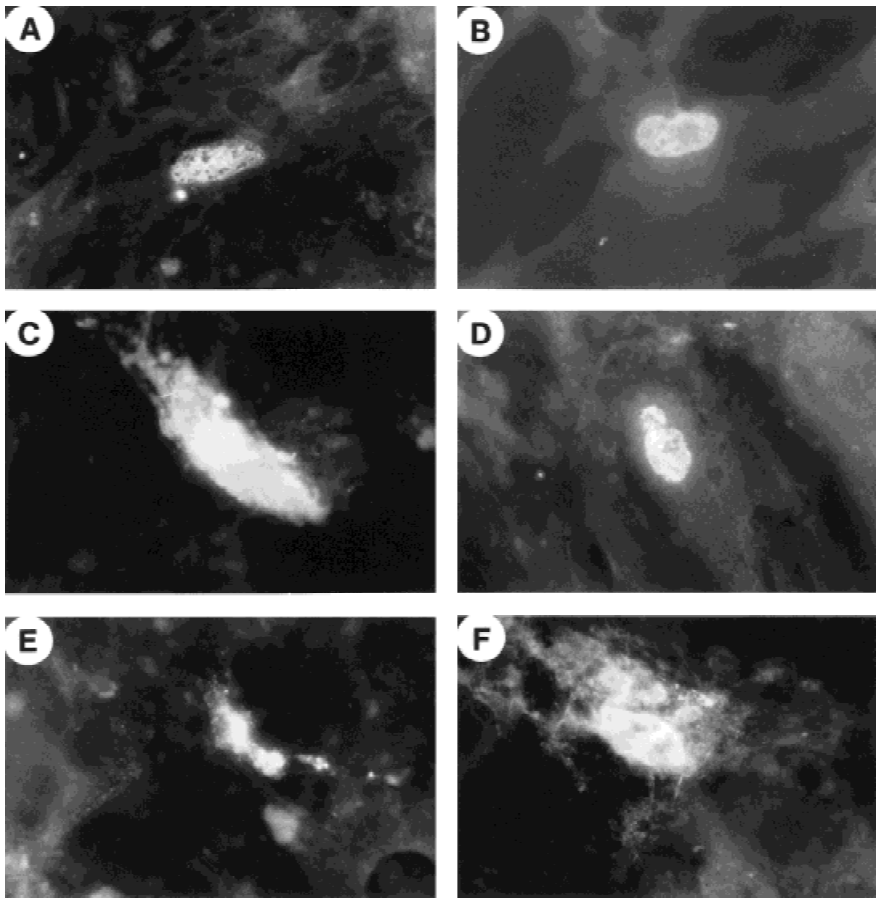


Fig. 5. EBV antigen expression demonstrated by immunofluorescence microscopy in the LMS-1 leiomyosarcoma cells cultured in vitro. EBV antigens included the latent antigens EBNA-1 **(A),** detected by monoclonal antibody (Advanced Biotechnologies Incorporated, Columbia, MD); the immediate-early antigen BZLF1 **(B);** the early antigens EA-D **(C);** and the late antigens p160 **(D),** gp125 **(E),** and gp350 **(F).** Magnification ×500.

EBNA-2, and LMP-1 (Fig. 6A–C); immediate-early antigen BZLF1 (Fig. 6D); early antigens EA-D and EA-R (Fig. 6E,F); and late antigens, including viral capsid antigens p160 and gp125, and membrane antigen gp350 (Fig. 6G–I). This repertoire is similar to EBV antigen expression and to immunostaining patterns that are typical of other EBV-infected lymphoid cell lines.

## DISCUSSION

Leiomyosarcomas are rare tumors of smooth muscle, but EBV-associated leiomyosarcomas are the second

TABLE II. Epstein-Barr Virus Protein Expression Detected by Immunofluorescence in the LMS-1 Leiomyosarcoma Cells Cultured in Vitro and in EL-1, a Human Lymphocyte Cell Line Derived by Immortalization of Umbilical Lymphocytes With Epstein-Barr Virus From LMS-1*

| EBV protein | LMS-1 | EL-1 (lymphocytes immortalized with EBV from LMS-1) |
|---|---|---|
| Latent antigens | | |
| EBNA-1 | | |
| Using monoclonal antibody | Positive (≤1%) | Positive (≤1%) |
| Using human serum | Indeterminate | Positive (≥80%) |
| EBNA-2 | Negative | Positive (~80%) |
| LMP-1 | Negative | Positive (~50% weak; ≤1% strong) |
| Early antigens | | |
| BZLF1 | Positive (≤1%) | Positive (≤1%) |
| EA-D | Positive (≤1%) | Positive (≤1%) |
| EA-R | Positive (≤1%) | Positive (≤1%) |
| Late antigens | | |
| p160 (VCA) | Positive (≤1%) | Positive (≤1%) |
| gp125 (VCA) | Positive (≤1%) | Positive (≤1%) |
| gp350 (MA) | Positive (≤1%) | Positive (≤1%) |

*Epstein-Barr virus (EBV) latent antigens were detected primarily with rare cells that were positive for the early replicative proteins, EA-D or EA-R, or for the late replicative proteins, viral capsid antigen (VCA) and membrane antigen (MA).

most prevalent malignancy of young persons with AIDS [Jenson et al., 1997b]. Similar smooth muscle tumors containing EBV have been reported in children following liver transplantation [Lee et al., 1995; Timmons et al., 1995]. The causal role of EBV in the development of these tumors is suggested by 1) identification of EBV in each cell of these tumors by in situ hybridization; 2) the clonality of EBV within each tumor, even for multiple tumors from the same patient; and 3) quantitative PCR amplification findings of approximately 4.5 EBV genome copies per cell [Jenson et al., 1997b; McClain et al., 1995]. In this report, we describe the characterization of in vitro cultured cells from a leiomyosarcoma of the thoracic spine from an HIV-infected young woman. These studies demonstrate that the cultured LMS-1 cells are EBV-infected smooth muscle cells that express both latent and lytic viral gene products with complete viral replication and release of infectious virions.

The smooth muscle origin of the LMS-1 cells was confirmed by immunostaining with cell type-specific antibodies (Table I). Greater than 90% of cells expressed smooth muscle actin and smooth muscle myosin by immunoperoxidase staining, whereas 25% of cells demonstrated very bright fluorescence to smooth muscle calponin and actin. All cells appeared to have the same morphology and growth characteristics. Thus, the differences between immunoperoxidase and immunofluorescence most likely reflect distinct sensitivities between the two methods. Nevertheless, the repertoire of tissue-specific markers expressed in LMS-1 cells indicates the mesenchymal origin and smooth muscle differentiation of these cells, consistent with the original tumor histopathology. Particularly relevant is the lack of expression of the endothelial cell marker factor VIII that would be expected in a mixed or multilineage mesenchymal cell population [Jaffe, 1982]. The absence of neural cell markers was expected, although the GalC marker is expressed in some endothelial and nonneuronal cell types [Kennedy, 1982; Schneider et al., 1986].

The LMS-1 cells expressed either the EBV B cell receptor CD21 or an antigen that cross reacts with certain anti-CD21 monoclonal antibodies. Three monoclonal antibodies that recognize different epitopes of CD21 were used in this study: 1) OKB7, an IgG-2b antibody that detects a 140,000-Da surface antigen and blocks C3d and EBV binding; 2) HB5, an IgG-2a monoclonal antibody that recognizes CD21; and 3) B2, an IgM monoclonal antibody specific for CD21. The presence of a CD21 epitope recognized by OKB7 but not HB5 or B2 suggests either that the smooth muscle cells may present cell surface CD21 differently than B lymphocytes or that OKB7 detects a related antigen. In this regard, a 200-kDa molecule has been detected in epithelial cells with HB5 and B2 antibodies [Young and Sixbey, 1988]. The discrepancy in size between CD21 and the 200-kDa molecule in epithelial cells presumably may be due to differences in posttranslational modification. Differences in posttranslational modification (e.g., glycosylation) also can affect protein folding and may explain the lack of recognition of CD21 by HB5 and B2 in smooth muscle cells. Alternatively, a novel EBV receptor has been found in human gastric carcinoma cells, supporting the possibility that smooth muscle cells similarly may express a distinct protein that cross reacts with OKB7.

The weak staining of CD21, or surface markers cross reacting with CD21 monoclonal antibodies, in leiomyosarcomas from HIV-uninfected patients compared with stronger staining in leiomyosarcomas from HIV-infected patients suggests that HIV may up-regulate CD21 expression or that EBV infection itself subsequently may up-regulate CD21 production [Jenson et al., 1997b; McClain et al., 1995]. The milieu resulting from HIV infection is associated with higher levels of

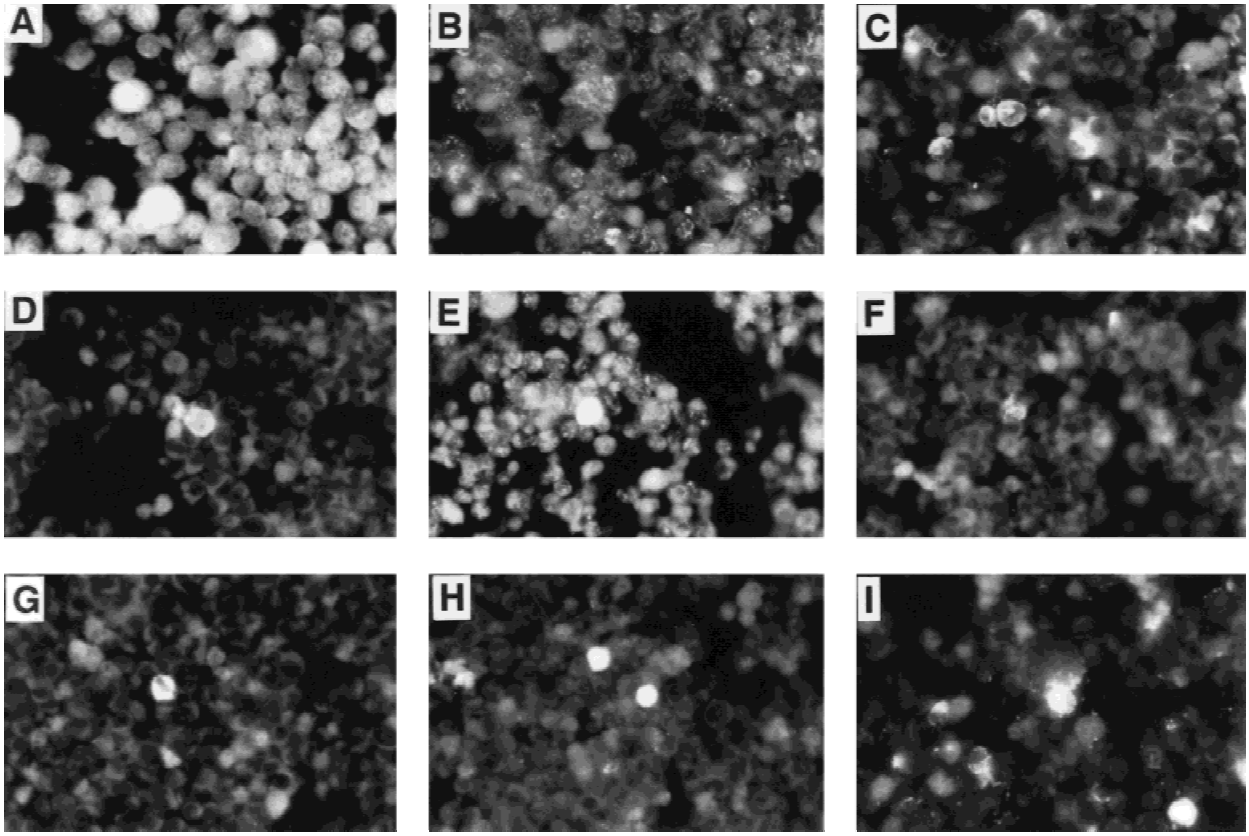


Fig. 6. EBV antigen expression demonstrated by immunofluorescence microscopy of EL-1, a cell line that was derived from human umbilical cord lymphocytes immortalized with EBV from the cultured LMS-1 leiomyosarcoma cells. The EBV antigens detected included latent EBNA-1 using polyclonal human serum **(A),** EBNA-2 **(B),** latent membrane protein-1 (LMP-1, **C**), the immediate-early antigen BZLF1 **(D),** the early antigens EA-D **(E)** and EA-R **(F),** the late antigens p160 **(G)** and gp125 **(H),** and the membrane antigen gp350 **(I).** The EL-1 lymphocyte cell line shows staining patterns typical of other EBV-infected lymphoid cell lines. Magnification ×500.

expression of CD21 and, consequently, may facilitate infection of these smooth muscle cells by EBV. Under such a scenario, HIV or the associated immunosuppression may facilitate initial EBV smooth muscle cell infection, but it is EBV that is directly responsible for malignant transformation to leiomyosarcoma.

Extensive analyses and characterization of virus gene expression in EBV-associated malignancies have been performed by other laboratories [for review, see Liebowitz and Kieff, 1993]. In most Burkitt's lymphoma (BL) tumors, EBV gene expression is limited to EBNA-1 [Rowe et al., 1987; Sample et al., 1991; Schaefer et al., 1991], but, in a small fraction of BL tumors, all six EBNA gene products and the latent membrane proteins (LMP-1 and LMP-2) are expressed [Kerr et al., 1992; Rowe et al., 1987] Occasionally, latency is disrupted, and lytic gene expression is seen in BL cell lines. In nasopharyngeal carcinoma (NPC) and NPC-derived tumors propagated in nude mice, EBNA-1 is found in virtually all cases, but only a few of the tumors express LMP-1 [Fahraeus et al., 1988; Tugwood et al., 1987; Young et al., 1988]. Recent reports describe the expression of BZLF1 and other lytic genes in NPC tissues, suggesting an abortive lytic cycle in these tumors [Cochet et al., 1993; Martel-Renoir et al., 1995]. However, little is known about virus expression in vitro in NPC cells, because cell lines have been difficult to establish. In addition to BL and NPC, EBV gene expression has been studied in gastric adenocarcinomas [Osato and Imai, 1996; Sugiura et al., 1996], Hodgkin's disease [Chiang et al., 1996], and AIDS-related lymphomas [Carbone et al., 1996]. Virus gene expression in these three malignancies also is limited generally to latent genes.

The cultured LMS-1 cells described in this report expressed numerous EBV-encoded antigens, including latent antigen EBNA-1, the immediate-early antigen BZLF1, the early antigens EA-R and EA-D, and the late replicative products (viral capsid antigen p160, gp125, and membrane antigen gp350). EBNA-2 was not found (data not shown), but it has been found in approximately 50% of cells of two leiomyosarcomas stained for EBNA-2 [Lee et al., 1995]. The levels of EBV antigen detected in smooth muscle cells may be due to lower production of EBV proteins resulting from fewer genome copies per cell (4.5 EBV genomes per smooth muscle cell [Jenson et al., 1997b] compared with 10–400 EBV genomes per B lymphocyte [Sternås et al., 1990]) or to different sensitivity of these reagents, which have been developed and selected for de-

tection of EBV infection in B lymphocytes, for EBV infection in smooth muscle cells. It is conceivable that a subpopulation of latently EBV-infected smooth muscle cells undergoes spontaneous EBV reactivation in vivo and in vitro. Alternatively, lytic gene expression in cultured LMS-1 cells may result from changes in the cellular environment secondary to explanation and in vitro growth that are more amenable to lytic viral replication. Lytic replication may result in the loss of latently infected cells and, thus, decreased expression of EBER and EBV proteins in the cultured cell population. In this regard, normal smooth muscle cells have been shown to undergo extensive changes in their differentiated phenotype when they are grown in vitro, but it is not known presently whether similar changes occur in transformed cells [Owens, 1995].

The absence of latent proteins other than EBNA-1 is similar to in vitro infection of SVK-CR2 cell line infected with the Akata strain of EBV [Li et al., 1992]. Immortalization of primary lymphocytes (the EL-1 lymphocyte cell line) using supernatant from cultured LMS-1 cells from each passage tested (passages 2 through 6) is conclusive evidence of complete viral replication in smooth muscle cells. Infective EBV was obtained through each of the multiple passages tested for transforming virus. The permissive nature of EBV replication in LMS-1 cells suggests that EBV may replicate in smooth muscle cells in vivo. Recent studies by others have shown that the stable maintenance of EBV and, presumably, latent gene expression in SVK-CR2 cells may require an undifferentiated cellular environment [Knox et al., 1996]. The cultured LMS-1 cells exhibited mature smooth muscle cell markers with lytic viral replication. It will be of interest to determine whether the state of differentiation of a smooth muscle cell dictates which EBV genes (i.e., latent versus lytic) are expressed in infected cells, such as the restriction of EBV replication to terminally differentiated squamous epithelium in oral hairy leukoplakia [Young et al., 1991]. At this time, however, these studies in cultured leiomyosarcoma cells are unable to be performed because no specific markers for smooth muscle precursor cells are available currently.

This is the first characterization of the nature of EBV infection in smooth muscle cells and the first documentation of lytic EBV replication in smooth muscle cells. Characterization of EBV infection in smooth muscle cells is important in augmenting our understanding of the viral and cellular differences of EBV infection in different cell types. The association of EBV with a tumor outside of traditional cell targets provides a unique opportunity to study a different facet of the oncogenic role of EBV. Study of these rare tumors provides a new window to understanding the aspects of viral immunology that are responsible for controlling development of these EBV-associated tumors in immunocompetent persons, especially toward understanding the viral interrelationships of EBV and HIV.

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